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A RNA helix-destabilizing protein is a major component of *Artemia salina* nuclear ribonucleoproteins

(heterogeneous nuclear ribonucleoprotein structure/glycine-rich nuclear protein/RNA–protein complexes)

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ABSTRACT **A major component of 30S heterogeneous nuclear ribonucleoprotein (hnRNP) particles from *Artemia salina* is HD40, a protein that has been characterized as a RNA helix-destabilizing protein [Marvil, D. K., Nowak, L. & Szer, W. (1980) *J. Biol. Chem.* 255, 6466–6472; Nowak, L., Marvil, D. K., Thomas, J. O., Boublik, M. & Szer, W. (1980) *J. Biol. Chem.* 255, 6473–6478]. HD40 binds to and disrupts the secondary structure of nuclear RNA fragments isolated from 30S hnRNP with a stoichiometry of one protein per 10–12 nucleotides. The addition of HD40 in excess of this ratio results in the formation of bead-like HD40–nuclear RNA complexes that are similar in properties and appearance to native 30S hnRNP particles. The heterogeneous nuclear RNA (hnRNA) in the HD40–hnRNA complexes is unstacked and unfolded to about the same extent as the RNA in the native 30S hnRNP particles. HD40 is strikingly similar in molecular weight (40,000) and amino acid composition (no cysteine, high glycine, presence of dimethylarginine, and blocked NH_2 terminus) to eukaryotic hnRNP proteins isolated from many cell types. HD40 can be separated into three isoelectric species with basic pIs, which appear to be posttranslational modifications of a single polypeptide chain.**

As it is being transcribed, heterogeneous nuclear RNA (hnRNA) associates with proteins to form heterogeneous nuclear ribonucleoprotein (hnRNP) complexes (1, 2). These complexes can be seen in electron micrographs of chromatin undergoing transcription as globular "beaded" structures along the length of newly transcribed RNA (3–5). When nuclei are incubated at pH 8.0, nuclear RNP particles that sediment at 30–50 S are released, presumably as a result of the cleavage of nuclease-sensitive regions between the "beads" (6–8).

Since hnRNP are probably involved in the processing of primary transcripts and in the generation of mRNA molecules, there is considerable interest in their structure and in the function of individual hnRNP proteins. There is a growing body of evidence that hnRNPs from many eukaryotic cells contain a class of basic polypeptides in the 30,000–40,000 molecular weight range that forms the core of the monomeric 30–50S hnRNP particles and, thus, may play a structural role in the organization of hnRNPs (9–11). Proteins within this class, regardless of the cell of origin, are characterized by a high content of glycine, little or no cysteine, and the presence of the unusual amino acid dimethylarginine. There appear to be only a few polypeptides having different primary structures within this class, but extensive modifications after translation give rise to multiple protein bands upon isoelectric focusing (9, 11). In rat brain and in mouse ascites cells, the NH_2-terminal amino acid of all of the glycine-rich proteins is blocked (11, 12).

We have obtained from extracts of dormant (undeveloped) cysts of the brine shrimp *Artemia salina* a homogeneous protein that is strikingly similar in amino acid composition and molecular weight to this class of hnRNP proteins (13, 14). The protein, designated HD40 to indicate that it is a helix-destabilizing protein with a molecular weight of 40,000, binds to single-stranded (but not double-stranded) synthetic and natural polynucleotides and disrupts their residual secondary structure. The polynucleotides are maximally unwound at a stoichiometry of one HD40 per 12–15 nucleotides. The addition of HD40 in excess of this ratio results in the formation of globular structures or "beads" along the entire polynucleotide strand. These complexes are reduced in contour length with respect to the unfolded complexes obtained at a 1:12 stoichiometry, have an appearance similar to the beaded structure of hnRNPs, and are largely protected from degradation by nucleases (13, 14). For these experiments, HD40 was purified on a preparative scale from extracts in which the nuclei had been broken.

In this communication we show that HD40 is a major component of 30S RNP particles extracted from intact nuclei of developed *A. salina* cysts and that homogeneous HD40 binds to hnRNA fragments extracted from the nuclei of developed cysts, forming complexes that are similar in properties and appearance to native 30S hnRNP particles. Development of the cysts to the prenauplius stage involves extensive RNA and protein synthesis but no cell division (15). In undeveloped cysts HD40 is found primarily in the cytoplasm (13), whereas in developed ones a significant amount of the protein is present in the nucleus. The overall amount of HD40 remains fairly constant during development.

MATERIALS AND METHODS

Purification of HD40 from Developed Cysts. *A. salina* cysts (San Francisco Bay Brand, obtained from Metaframe Corp., Newark, CA) were pretreated and grown to the prenauplius stage (referred to as developed) as described (16). HD40 was isolated from developed *Artemia* by the procedure employed for the purification of the protein from undeveloped cysts (13). As judged by $NaDodSO_4$/polyacrylamide gel electrophoresis, the protein was better than 90% pure after step 6. When this protein was mixed with step-6 HD40 from undeveloped cysts, a single band was obtained by this electrophoretic method.

Preparation of Antibodies Against HD40. HD40 (1.0 mg, 95% pure; obtained from undeveloped cysts) was electrophoresed on a $NaDodSO_4$/polyacrylamide slab gel (4 × 140 × 180 mm). A horizontal strip of gel corresponding to the center of the HD40 band was excised, ground in a mortar, and mixed with

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Abbreviations: hn, heterogeneous nuclear; RNP, ribonucleoprotein.

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an equal volume of incomplete Freund's adjuvant. The resulting suspension was used directly for immunization of New Zealand White rabbits. The injection (100–200 μg of protein) was repeated after 3 and 8 wk. Thereafter, booster injections (15–25 μg of protein) were given 10 days prior to bleeding. Immunoglobulins were purified from the antiserum by ammonium sulfate precipitation or by DEAE-Sephadex chromatography (17), or both, and were dialyzed against a buffer containing 10 mM Tris·HCl, pH 7.8/100 mM KCl. When examined by the double-immunodiffusion assay with HD40 (2 μg) purified from both undeveloped and developed cysts, the anti-HD40 antibodies (10 μl) gave single confluent precipitin lines of equal intensity.

Extraction of hnRNP Particles. *A. salina* cysts (100 g), pretreated and developed to the prenauplius stage as described (16), were suspended in 1.2 liters of buffer A (20 mM Tris·HCl, pH 7.0/20 mM NaCl/1.5 mM $Mg(C_2H_3O_2)_2$/1.0 mM dithiothreitol/0.25 M sucrose) and homogenized with a Thomas Teflon homogenizer (three strokes; loose pestle). After filtering through four layers of cheesecloth and glass wool, the homogenate was centrifuged at 1000 × *g* for 10 min. The pellet was suspended in 50 ml of homogenizing buffer, the mixture was centrifuged at a low speed (30–50 × *g*) for 2 min to remove yolk granules, and the supernatant was centrifuged at 1000 × *g* for 10 min. The pellet was then resuspended in 25 ml of buffer A containing 100 mM NaCl, the mixture was centrifuged at 30–50 × *g* for 2 min, and the supernatant was centrifuged at 1000 × *g* for 10 min; this was repeated until the contamination with yolk granules was less than 10–20%. The pellet was resuspended in buffer A and washed twice, with a 10-min 1000 × *g* centrifugation between washings. The resulting nuclei, which were intact as judged by phase-contrast microscopy, were free of cytoplasmic debris. To extract the RNP particles, the nuclei were suspended in 20 ml of buffer B (buffer A at pH 8.0 containing no sucrose), the pH of the suspension was adjusted to 8.0, and the nuclei were stirred gently at 4°C for 12–14 hr. The nuclei were pelleted and reextracted twice more, for 2 hr each time. Of the nucleic acid present in the extract, 95–100% was RNA. All three extracts were combined and centrifuged for 4 hr at 250,000 × *g*. The pellet was resuspended and sedimented in a 10–30% (wt/vol) sucrose gradient at 23,000 rpm for 15 hr in an SW41 rotor (see Fig. 1*A*). RNA was extracted from sucrose gradient-purified hnRNP or directly from the pH 8.0 extract by the phenol/chloroform procedure (18).

Chemical and Physicochemical Analyses. Protein was determined by the Bio-Rad method (19), DNA was determined by a modified diphenylamine test (20), and RNA was assayed by absorbance at 260 nm (ε_{260} = 8100) or by a modified orcinol test (21). Amino acid analysis was performed in a Durum D-500 automatic analyzer after hydrolysis of the protein sample (0.1–0.2 nmol) for 21 and 48 hr (22). NH_2-terminal amino acid determination was carried out by a manual Edman degradation by using amino acid analysis (5–7 nmol of protein) to identify any cleaved derivatized amino acid (23). Circular dichroic (CD) spectra of samples containing 10 mM Tris·HCl, pH 7.4/50 mM NaCl were recorded with a Cary 61 spectropolarimeter equipped with jacketed thermostated cells (5-mm path length; 0.5-ml vol). Electron microscopy is described in the legend to Fig. 4.

Electrophoretic Analyses. $NaDodSO_4$/polyacrylamide gel electrophoresis was with 10% (wt/vol) slab gels (1.2 × 140 × 180 mm) as described (24). Isoelectric focusing (25) was carried out on 7.5% (wt/vol) polyacrylamide column gels (6 × 85 mm). Crossed immunoelectrophoresis was carried out by a modification of the procedure of Converse and Papermaster (26) as described by Chua and Blomberg (27).

RESULTS

Protein HD40 Is a Major Component of Nuclear RNP Particles in Developed Cysts. HD40 is found mainly in the cytoplasm of undeveloped *A. salina*, but after 18–20 hr of development, a considerable amount of the protein appears in the nucleus as revealed by immunoelectrophoresis (28). The protein isolated from the developed cysts was identical to the HD40 previously purified from undeveloped cysts (13) as judged by a number of criteria. They were antigenically identical, and their molecular weights and amino acid compositions were the same (including the absence of cysteine and the presence of five residues of dimethylarginine and 75 residues of glycine; see figure 2 and table II of ref. 13). They both bound to single-stranded DNA-agarose (and RNA-cellulose) columns and eluted with buffers containing 0.5 M NaCl. Titration of single-stranded polynucleotides with the protein from developed cysts (not shown) resulted in progressive unwinding of their secondary structure followed by the formation of globular structures along the nucleic acid strand, exactly as described for the protein from undeveloped cysts (14). The yield of HD40 was about the same from either developed or undeveloped cysts—12–15 mg of 90–95% pure protein per 500 g of cysts.

When nuclei of developed cysts were extracted with a low-salt, pH 8.0 buffer [a treatment that in higher eukaryotic cells leads to the formation of nuclear RNP particles that sediment at 30–50 S (6–12)], a RNP complex sedimenting at about 30 S was obtained (Fig. 1*A*). The protein/RNA ratio of this 30S material was about 6:1; contamination by DNA varied (0–3%) in several preparations. When the 30S material was visualized by electron microscopy, it consisted of particles with a diameter of about 15 nm (see Fig. 4). Analysis of the 30S-peak proteins by electrophoresis (Fig. 1*B*) revealed a major 40,000 molecular weight band that migrated at the same rate as authentic HD40, several prominent bands in the 90,000–100,000 molecular weight range, and a number of minor bands. In several preparations, the amount of the 40,000 molecular weight protein, identified antigenically as HD40 (Fig. 1*C*), varied from 35% to 60% of the total 30S-particle protein. The 30S particles were not contaminated with any significant amount of low molecular weight proteins, such as histones or ribosomal proteins. Most of the proteins in the 30S fraction also sedimented near the top of the gradient, which may have been due to degradation of the particles. The 30S particles were sensitive to increased salt concentration. In 0.2 M NaCl a RNP complex sedimenting at about 25 S was seen in the gradient (Fig. 1*A*), but in 0.8 M NaCl nearly all of the material, including more than 90% of HD40, sedimented close to the top of the gradient. A detailed account of the effect of the ionic environment on the 30S particles will be published elsewhere.

The major 40,000 molecular weight protein of the 30S particles was immunologically crossreactive with HD40, as shown by crossed immunoelectrophoresis (Fig. 1*C*). In this technique (27), the polypeptides of the 30S peak resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis in the first dimension (Fig. 1*B*), were electrophoresed in the second dimension into an agarose gel containing anti-HD40 antibodies. A large precipitin arc was formed at the position of HD40, with three or four smaller arcs from antigenically related proteins in the 25,000–40,000 molecular weight range (Fig. 1*C*). The pattern produced by crossed immunoelectrophoresis of nearly homogeneous HD40 is shown in Fig. 1*D*. The precipitin arcs at the position of HD40 in Fig. 1 *C* and *D* have similar appearances. They consist of three overlapping peaks: two major ones and a faint, fast-migrating one. When nearly homogeneous HD40 was added to the 30S material and the mixture was subjected to

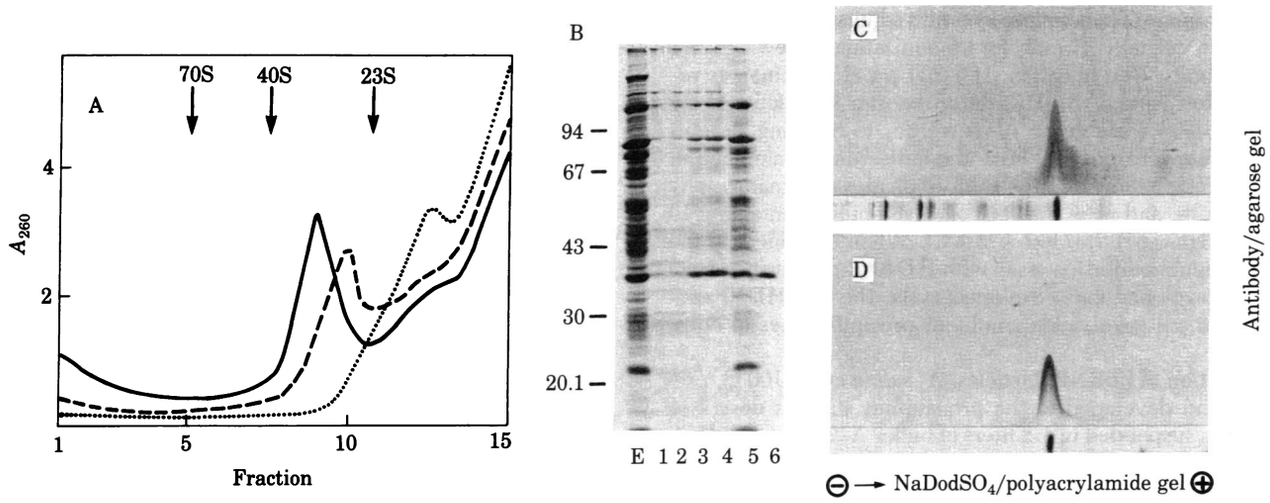

FIG. 1. Isolation and analysis of nuclear RNP. (*A*) Sucrose gradient sedimentation of the nuclear extract in buffer B at NaCl concentrations of 0.02 M (——), 0.2 M (– – –), and 0.8 M NaCl (...). (*B*) $NaDodSO_4$/polyacrylamide gel electrophoresis of the sucrose gradient fractions obtained at 0.02 M NaCl. Lanes: E, pH 8.0 extract; 1–5, sucrose gradient fractions 1, 2, 9, 10, and 15, respectively; 6, purified HD40. The positions of molecular weight markers ($M_r \times 10^{-3}$) are indicated on the left. (*C*) Crossed immunoelectrophoresis of the 30S peak material. A longitudinally cut unstained strip of the $NaDodSO_4$/polyacrylamide gel of fraction 9 in *A* (0.05 ml) was electrophoresed at a right angle into the anti-HD40 antibody-containing gel. An identical strip of the first-dimension gel was stained and aligned with the second-dimension gel. (*D*) Crossed immunoelectrophoresis of step 6 HD40 (3.0 μg).

crossed immunoelectrophoresis, a single but enlarged precipitin arc, consisting of three overlapping peaks was formed at the expected position (not shown). The antigen used to raise anti-HD40 antiserum contained three isoelectric species (see below). The three overlapping precipitin arcs produced by both the purified HD40 and the 30S RNP protein may result from the interaction between the three isoelectric species of HD40 and antibodies against specific antigenic determinants on each of the three species.

HD40 Contains Three Isoelectric Species and a Blocked NH_2-Terminal Amino Acid. HD40 was resolved by electrofocusing into two major and one minor ionic species comprising 54%, 37%, and 8–10% of the total protein applied (determined by densitometry of the gels of Fig. 2) with pIs of 8.1, 7.8, and 7.6 (±0.1 unit), respectively. The pI values and relative amounts of each species of HD40 were the same in undeveloped and developed cysts. Incubation of HD40 with bacterial phosphatases had no effect on the pattern produced by electrofocusing.

No NH_2-terminal amino acid could be detected when HD40 was analyzed by the Edman degradation (23), even when amounts of protein sufficient to detect a free NH_2-terminal residue from only the minor ionic species (pI of 7.6) were used. Identical results were obtained whether the protein was from undeveloped or developed cysts.

The identical molecular weights of the three ionic species of Fig. 2, their copurification through a number of steps during the isolation of HD40 (including affinity chromatography), and the blocked NH_2 terminus and lack of cysteine in all three of them suggest that the three species arise as a result of modification of a single polypeptide chain.

HD40 Unwinds Nuclear RNA. RNA isolated by phenol extraction from 30S particles sedimented in an isokinetic sucrose gradient as a broad peak with a center around 6–9 S. Peak fractions had an ellipticity of 12–13 × 10^3 degrees cm^2/dmol at 23°C (Fig. 3); at 80°C the ellipticity was reduced by about 70% to 3.5–4 × 10^3 degrees cm^2/dmol. Titration of hnRNA with HD40 at 23°C resulted in a 60% decrease in the ellipticity of the RNA to 5.1 × 10^3 degrees cm^2/dmol at the titration end point (Fig. 3 *Insert*). This large decrease in ellipticity, which was accompanied by a small red shift, indicates an unfolding of the secondary structure of the RNA equivalent to thermal denaturation at 60–65°C (cf. Fig. 3, curves 2 and 4). The decrease in ellipticity leveled off at a molar ratio of approximately one protein per 10.3 nucleotides of RNA (Fig. 3). These results are very similar to the reported disruption of the secondary structure of synthetic and natural single-stranded ribo- and deoxyribonucleotides by HD40 (14).

In intact 30S particles, the ellipticity of the RNA was very close to that of the RNA–HD40 complex, being reduced by 65% from the ellipticity of the protein-free RNA (Fig. 3). Exposure of either intact 30S RNP or RNA–HD40 complexes to increasing concentrations of NaCl or $MgCl_2$ brought about a progressive increase in the ellipticity of the RNA (not shown).

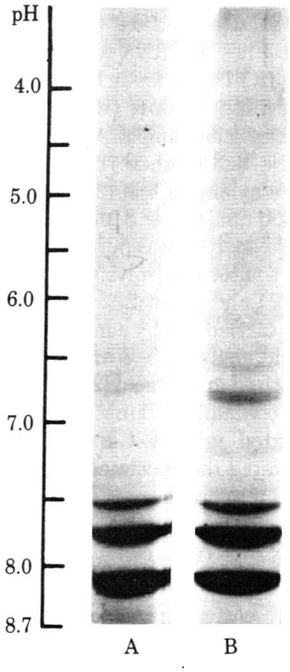

FIG. 2. Electrofocusing of step 6 HD40 (30 μg). Lanes: A, protein isolated from undeveloped embryos; B, protein from developed embryos.

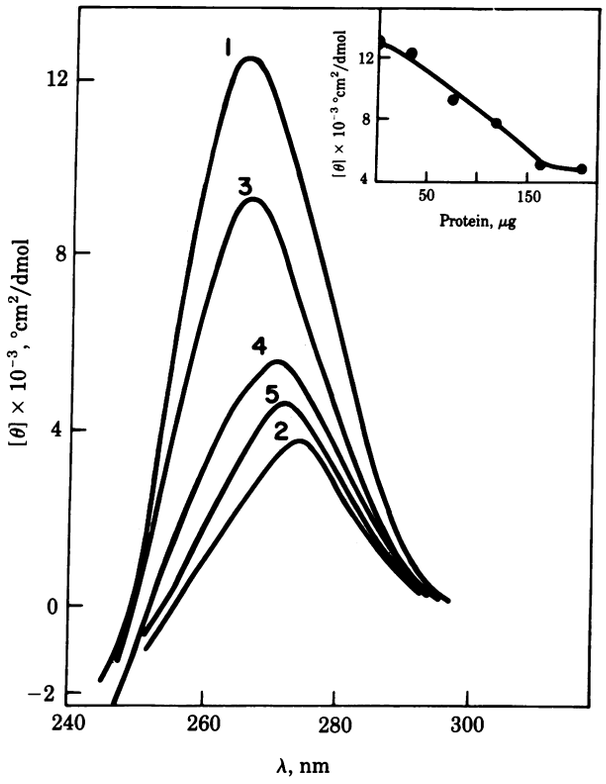

FIG. 3. Circular dichroic spectra of RNA from 30S nuclear RNP particles, RNA–HD40 complexes and native 30S nuclear RNP. Curves: 1 and 2, RNA (42.3 nmol of nucleotide) at 23°C and 80°C, respectively; 3 and 4, RNA (42.3 nmol of nucleotide) in the presence of 75.0 and 164.2 μg of HD40, respectively (4 represents the approximate end point of titration); 5, 30S RNP (31.8 nmol of nucleotide). (*Inset*) The effect of HD40 on the maximum ellipticity of nuclear RNA.

Electron Microscopy. Electron microscopic images of native 30S particles (Fig. 4*A*) and the complexes reconstituted from HD40 and hnRNA fragments isolated from the pH 8.0 nuclear extract (Fig. 4*B*) imply an overall structural similarity. The HD40–hnRNA complexes reconstituted with HD40 from either developed or undeveloped cysts are undistinguishable. Although both native and reconstituted particles show some degree of polydispersity, the major population consists, in either case, of roughly globular particles with a diameter of about 15 nm. In the reconstituted complexes (Fig. 4*B*), there are also structures that are composed of "strings" of globular particles. These presumably were formed from larger hnRNA fragments isolated from the pH 8.0 extract. Similar "beads-on-a-string" structures are seen in complexes formed between HD40 and single-stranded φX174 DNA (Fig. 4*C*; ref. 14) and coliphage MS2 RNA (14). In shadowed preparations mounted with the high resolution anthrabis method (14), the globular structures appear larger (about 20 nm) than in the negatively stained structures (Fig. 4), presumably due to the shadowing. Both highly folded beaded structures and more open complexes are seen in the same preparation of φX174 DNA-HD40 complexes (Fig. 4*C*). This suggests, as pointed out before (14), that the folding of the HD40–RNA complexes into compact "beaded" structures, which occurred at HD40/nucleotide molar ratios greater than about 1:10, is a cooperative process. On the other hand, the initial unfolding of RNA by HD40, which was complete at an HD40/nucleotide ratio of about 1:12, shows little cooperativity (14).

DISCUSSION

In this paper we present evidence that HD40, previously characterized as a helix-destabilizing protein with the capacity to form bead-like structures along the nucleic acid strand, is a major component of the 30S RNP particles obtained by standard hnRNP extraction methods from the nuclei of developed *A. salina* embryos. The physical properties of HD40, its nuclear location, the protection that it provides RNA from degradation by nucleases (13, 14), and the similarities between the 30S particles and the reconstituted HD40–hnRNA complexes (Figs. 3 and 4) are all consistent with a structural role for this protein in the formation of the hnRNP complex. Many of the physical properties of HD40 are strikingly similar to those of the core hnRNP proteins that have been isolated from a number of other eukaryotic cells. These include a characteristic amino acid composition (high glycine, no cysteine, dimethylarginine), a blocked NH_2 terminus, basic pIs (7.5–8.5) and a molecular weight range of 30,000–40,000 (9–11). In mammalian cells, the core hnRNP particles contain several glycine-rich basic proteins with mo-

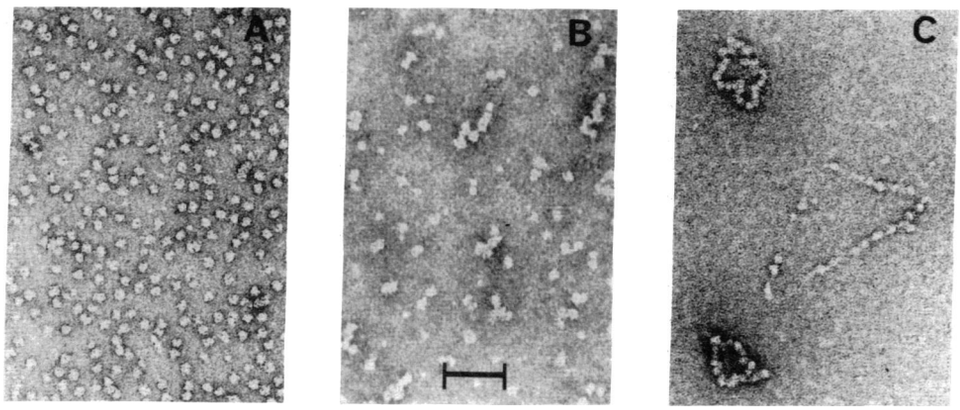

FIG. 4. Electron micrographs of 30S nuclear RNP particles and complexes of HD40 with nuclear RNA or single-stranded φX174 DNA. (*A*) A sample of RNP from the 30S peak (Fig. 1*A*) was fixed with 0.1% glutaraldehyde for 10 min at room temperature, applied to a Bio-Gel A-1.5 m column and eluted with 10 mM Tris·HCl/10 mM NaCl, pH 7.5. A sample from the void volume was collected directly on a carbon-coated grid. (*B*) Complexes between HD40 (90 μg/ml) and RNA obtained from the pH 8.0 nuclear extract by phenol extraction (4.5 μg/ml; one HD40 per six nucleotides) were formed in 10 mM Tris·HCl/10 mM NaCl, pH 7.5. After a 10-min incubation at room temperature, the complex either was fixed with 0.1% glutaraldehyde for 10 min at room temperature and then applied to a carbon-coated grid or was applied to a carbon-coated grid without fixation. There is no difference in appearance between glutaraldehyde-fixed and unfixed samples. (*C*) Complexes between HD40 and φX174 single-stranded DNA were prepared as in *B*, with φX174 DNA in place of RNA; as in *B*, fixed and unfixed complexes have the same appearance. All samples were contrasted with 0.5% uranyl acetate. (Bar = 100 nm.)

lecular weights of 30,000–40,000. In *A. salina*, a primitive crustacean (29), a single protein, HD40, is the most abundant protein within this molecular weight range (Fig. 1). *Physarum* tacean (29), a single protein, HD40, is the most abundant protein within this molecular weight range (Fig. 1). *Physarum polycephalum* also contains a single nuclear protein with these properties (30).

The RNA in the 30S particles is unfolded and unstacked to a considerable extent as shown by the circular dichroism data of Fig. 3, and the RNA in the reconstituted HD40–hnRNA complexes is unwound to a similar degree (Fig. 3). Analogous observations were reported for the hnRNA in 40S hnRNP particles from rat liver (9, 31). We have shown that HD40 binds to polynucleotides in a stepwise process (14). As polynucleotides (e.g., coliphage MS2 RNA and single-stranded ϕX174 DNA) are titrated with HD40, they progressively lose their residual secondary structure, becoming increasingly unstacked until a stoichiometry of about 1 HD40 per 10–15 nucleotides is reached. When additional HD40 is added, bead-like structures begin to form along the entire polynucleotide chain (Fig. 4*C*). The appearance of the "beads" is not accompanied by any further changes in the circular dichroic spectrum, indicating that the RNA in the beads remains unstacked. The contour lengths of the fully "beaded" complexes obtained at a stoichiometry of about 1:6 are 50–70% shorter than the maximally unfolded structures obtained at a stoichiometry of about 1:12 (28). These observations suggest that the beads function to compact the RNA without an increase in secondary structure, perhaps as a coiled RNA–protein complex.

Comparative investigations of the properties of HD40 purified from either dormant or developed cysts provide no evidence for any detectable modifications during 18–20 hr of development. Nor is there any significant change in the overall amount of the protein during this period. In undeveloped cysts, HD40 is found primarily in the cytoplasm; presumably, the protein appears in the nucleus concomitant with the resumption of transcriptional activity during development. Preliminary evidence suggests that, in undeveloped cysts, at least part of the HD40 may exist in association with translationally inactive poly(A)$^+$mRNP particles (28). Although this evidence is suggestive, the present methods for isolating mRNPs are not sufficiently specific to permit the unambiguous identification of a protein that copurifies with cytoplasmic poly(A$^+$)mRNA as a component of mRNP. Even in developed cysts, the supernatant following the removal of the nuclei contains a significant amount of HD40 as shown by crossed immunoelectrophoresis (unpublished observations). It remains to be established whether or not HD40 is involved in cytoplasmic RNA metabolism in *A. salina*, because its presence in the cytoplasm could be due to nuclear lysis and leakage [cf. early investigations on the cytoplasmic or nuclear location, or both, of eukaryotic α-DNA polymerase (32)].

Note Added in Proof. Peptide maps produced by partial cleavage of the individual isoelectric species of Fig. 2 with papain, chymotrypsin, and *Staphylococcus aureus* V8 protease indicate that the isoelectric species are derived from a single polypeptide chain.

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